ABSORPTION AND EXCRETION OF COPPER ION DURING SETTLEMENT AND METAMORPHOSIS OF THE BARNACLE, BALANUS AMPHITRITE NIVEUS [1]

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For hundreds of years ships have been sheathed with metallic copper or coated with copper-containing paints to prevent or minimize surface fouling. Sheathing is clearly subject to mechanical failure and once breached exposes the entire structure to attack. Copper-containing paints are effective only as long as enough toxic substance leaches from the coating to establish and maintain lethal concentrations within the barrier layer of water through which members of the fouling complex must approach the structure. These coatings gradually decrease in effectiveness during their service life, because the diffusion gradients necessary to sustained leaching are at best temporary. Millions of dollars annually and countless hours of ship time are devoted to protecting hulls from settling barnacles.

The general biology of the fouling problem, with primary emphasis upon the complex of fouling organisms, has been intensively studied in this laboratory for several years. It is the object of this communication to describe the pathways of copper absorption, circulation, and elimination by settling barnacles, and to discuss the mode of action of this toxic substance.

In a previous paper (Bernard and Lane, 1961) we have described the early stages in metamorphosis of *Balanus amphitrite niveus*. Histological localization of copper in developmental stages was determined by the dithio-oxamide (D.T.O.) method of Okamoto *et al.*, described by Gomori (1952). Specimens representing all the settling stages from normal sea water were fixed in absolute ethanol, stained for copper and then lightly stained with hematoxylin and eosin. Whole mounts, together with serial sections cut 5 and 10 microns thick in various planes of orientation, were prepared. The same procedure was repeated for specimens which had been exposed to 200 mg./kg. of copper ion in sea water for one hour.

Copper Distribution in Barnacles from Normal Sea Water

The concentration of copper ion in Gulf Stream sea water is approximately 0.005 milligrams per kilogram. *B. amphitrite niveus,* both planktonic larvae and attached forms, were collected in Biscayne Bay, where copper concentration is somewhat more variable, and were held in Millipore-filtered Gulf Stream sea water for one hour prior to histological preparation.

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A. *Planktonic cyprids*

Whole mounts of planktonic cyprids treated with D.T.O. show characteristic greenish-black precipitate of copper in the following sites (Fig. 1):

1. In ovoid to cuboidal vacuoles within the loose connective tissue underlying the postero-lateral carapace.
2. In shapeless masses in the connective tissues of the antero-ventral region.
3. In the epithelium lining the stomach.
4. In the epithelium lining the posterior two-thirds of the hind-gut.

The lateral copper-containing vacuoles of whole mounts fixed in absolute ethanol were quite consistent in pattern and number, varying from 47 to 54 in the eight specimens studied. The size ranged from 2.4–3.5 microns × 4.0–9.5

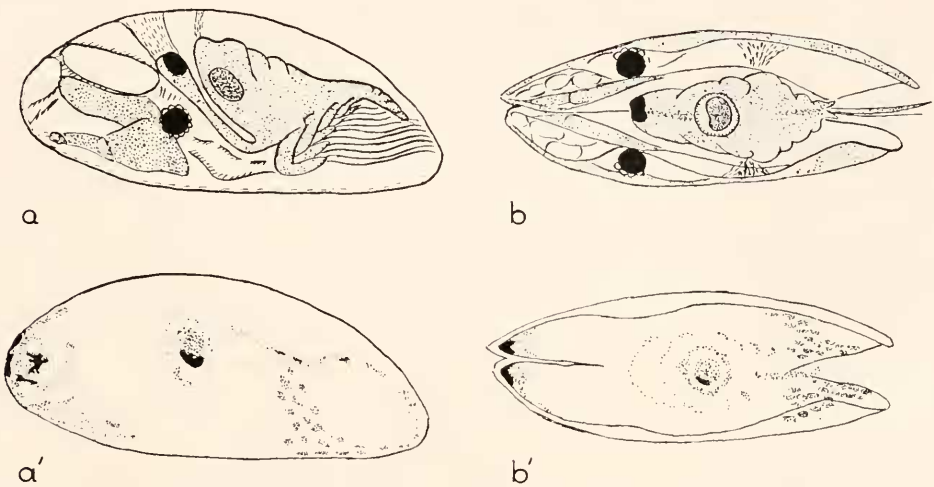

FIGURE 1. Distribution of copper ion in planktonic cyprids from normal sea water. a, a′: cyprid morphology, lateral aspect and corresponding areas of copper ion accumulation. b, b′: cyprid morphology, dorsal aspect and corresponding areas of copper ion accumulations.

microns. Copper is also concentrated within the loosely organized tissues of the anterior third of the animal, but masses of pigment granules normally present in the integument of the anterior region may mask discrete vacuolar arrangement. The outline of the stomach and of the posterior portion of the hind-gut were clearly marked by copper precipitate in whole mounts of D.T.O.-treated cyprids. A very heavy copper deposit was present in the food mass within the gut, and traces of copper-rich materials were distributed elsewhere in the gut of some animals. No copper was found in the anterior one-third of the hind-gut, in other thoracic structures, or in the digestive caeca.

While copper appears to be uniformly distributed through the stomach epithelium in whole mounts, histological sections reveal that its distribution is not uniform. The epithelial cells of the antero-ventral segment of the stomach contain more copper than cells in other areas. Granular copper deposits occur in the free edges of the cells, distal to the nucleus and mucous secretions. In cross-section the hind-gut is lined by 5 to 8 low pyramidal cells in which copper is restricted to a semi-lunar cap next to the lumen. Copper is found only in the posterior portion of the hind-gut, although there appears to be no histological difference between these areas and the region adjacent to the stomach.

Clarke (1947) observed that barnacle larvae concentrate copper from sea water and maintain internal concentrations higher than ambient. This is a common occurrence among invertebrates utilizing hemocyanin as a respiratory pigment. Secretion of copper into the lumen of the alimentary canal of barnacle larvae in normal sea water reveals a continual turnover, suggesting that the copper ion may participate actively in the normal physiology of these animals. A functional role is confirmed by the apparent storage of copper in postero-lateral vacuoles and in the loose connective tissue at the anterior of the body.

Since copper ions are moved against the concentration gradient it is reasonable to suggest an active process requiring metabolic work by the cells of the absorbing surface.

B. *Settled cyprids*

The sites of copper concentration in this stage are essentially the same as those of the planktonic cyprid. Anterior deposits of copper disappear with the degeneration and thinning of the connective tissue of this area following attachment. Secretion of copper by the hind-gut epithelium continues even though the entire tubular digestive system is displaced by ventral rotation of the thorax in this stage. Very light deposits of copper appear within the folds of the now inactive thoracic legs. The postero-lateral vacuoles are fewer in number than in the previous stage and are concentrated at the ventral surface. Movement of the

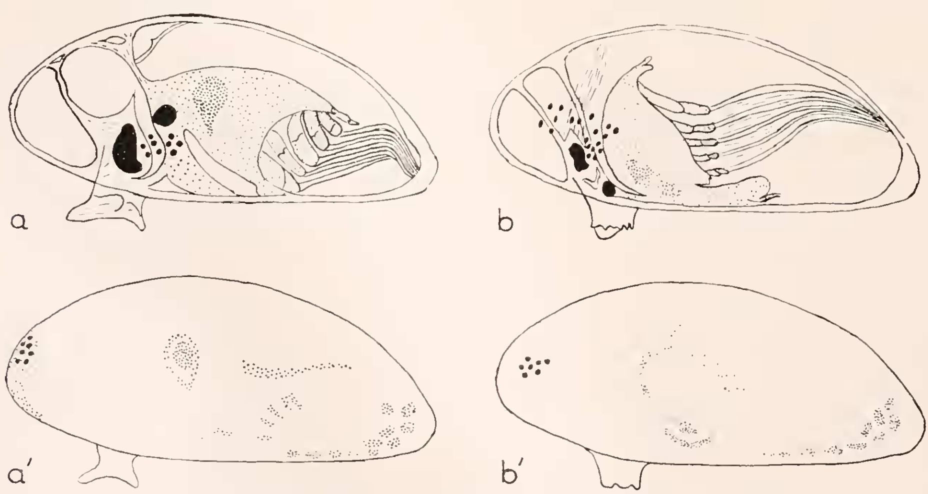

FIGURE 2. Distribution of copper ion in settled cyprid, lateral aspect. a, a′: structure shortly after attachment with corresponding sites of copper deposition. b, b′: structure just prior to decortication with corresponding sites of copper deposition.

general body tissues toward the ventral side during this stage probably accounts for this concentration (Fig. 2).

C. *Decorticated settlers*

Although a few individuals representing this stage contained small amounts of copper in isolated patches in the general parenchyma of the body, there was no consistent pattern of copper distribution.

D. and E. *Young barnacles, without and with shell plates*

No areas of copper concentration were noted, either in whole mounts or in histological sections of specimens taken during these stages.

Distribution of Copper in Barnacles Exposed to Excess Copper

Barnacles in the same metamorphic stages as above were exposed for one hour to Millipore-filtered Gulf Stream sea water to which 200 mg./kg. of cupric ion had been added. Although this concentration is 40,000 times that of normal sea water, it is yet sub-lethal. To dissolve more copper than this in sea water one must reduce the pH.

A. *Planktonic cyprids*

In spite of the great increase of available copper, the size and number of copper-containing vacuoles and the apparent concentration in connective tissues beneath the carapace are not appreciably augmented. These observations suggest either that copper reserves can only be built up over a longer period than the one-hour exposure period employed in this study, or that copper reserves are maximal in normal sea water and can not be increased further.

Deposits of copper in the epithelium of the hind-gut were much more numerous in these animals than in those from normal sea water. In barnacles from untreated sea water, copper deposits were present in approximately half the epithelial cells of the gut. The entire hind-gut epithelium, except for the portion nearest the stomach of specimens exposed to high concentrations of ambient copper, was dark with copper precipitate. Increased copper in the gut epithelium was not directly proportional to the increase in ambient copper concentration, but probably accurately reflected the increased absorption rate (Fig. 4).

In nature barnacle cyprids freely exchange copper with the medium. Established reservoirs within the body insure internal homeostasis in a medium which may fluctuate in copper content. The only sites where significant concentrations of copper normally occur are the copper-containing vacuoles and the epithelium of the hind-gut. In other areas, particularly the possible pathways from absorption sites to point of elimination, concentrations are too low to be revealed by the histochemical technique used.

When ambient copper concentrations were increased 40,000-fold, however, the amount of copper reaching the epithelium of the stomach and hind-gut appeared to exceed the excretory capacity of these cells. Copper accumulated, both in the excretory sites and in the areas of absorption. These sites of copper accumulations, then, reveal the absorptive surfaces and sites of final elimination from the organism and suggest pathways of distribution.

The surfaces of the cyprid which are covered with a thickened cuticle, *e.g.,* legs and antennae, contained no deposits of copper. If copper were, in fact, absorbed through these regions it was immediately translocated so no local concentration occurred. The surfaces of the thorax covered by thinner cuticle, however, contained particulate copper within the epithelial cells. The flattened epidermal cells covering the posterior part of the thorax were heavily laden with uniformly distributed copper deposits, particularly within dorsal folds. The cuboidal to columnar

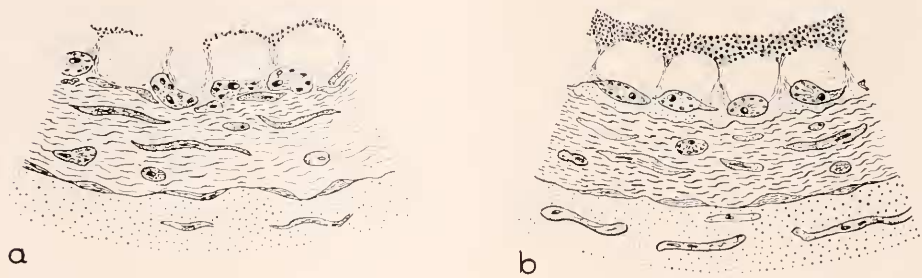

FIGURE 3. Secretion of copper ion by the gastric epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion.

FIGURE 4. Secretion of copper ion by hind-gut epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion for one hour.

epidermal cells over the anterior portions of the thorax were quite heavily marked by precipitated copper in treated specimens. Thin projections of the integument with little subepidermal connective tissue, *e.g.,* mouth parts or caudal flaps near the anus, contained copper in the integument as well as in some underlying cells. Diffuse accumulations of copper were also found in the wall of the fore-gut (Fig. 5, a and b).

The internal pathways through which copper circulates from absorptive sites on certain outer surfaces to excretory regions of the gut wall were not identified in this study. No consistent copper precipitate was observed in nerve or muscle fibers nor in the connective tissue underlying the epithelial layers. This suggests that the circulation of copper occurs in solution in blood and body fluids, where concentrations remain below detectable levels. Excretion is associated with local concentrations of copper.

Failure of the anterior third of the hind-gut to participate in copper exchange probably reflects a functional difference between this segment of the gut and all the rest of the alimentary canal (Fig. 5, c and d).

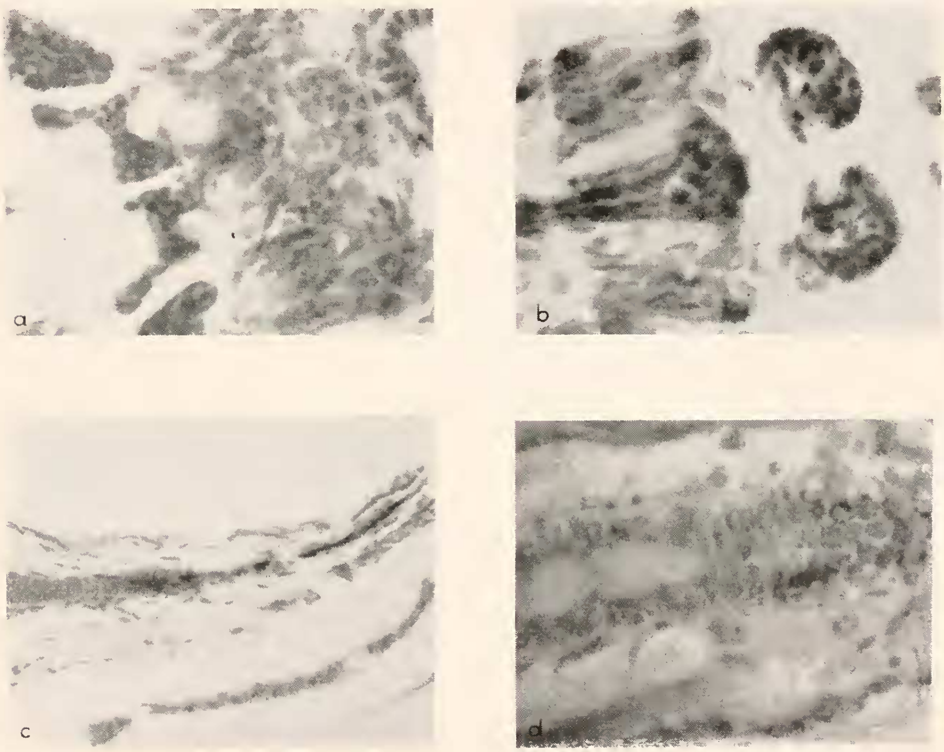

FIGURE 5. Copper precipitate in D.T.O.-treated cyprids exposed to 200 p.p.m. Cu^{++} in sea water. (a) In mouth parts and in epithelium of the fore-gut. (b) In hind-gut epithelium, anus, and anal flaps. (c) Along the posterior two-thirds of the hind-gut but not in anterior region. (d) At the point of transition between Cu^{++}-secreting and non-secreting zones.

No attempt was made to identify the mechanism of copper uptake, *i.e.*, whether it is by active ion transport or simple diffusion. This diffuse distribution of copper within absorbing cells supports the latter supposition. Active transport is suggested, however, by the polar distribution of precipitated copper within the cuboidal epidermal cells of the carapace. If the epicuticle of the carapace is impervious to ions, as is generally supposed, this copper must have been absorbed from water in the mantle cavity through the epithelium of the "mantle" and then accumulated in the epidermal cells of the carapace. The accumulations in mouth parts and anal flaps substantiate the observation that absorbed copper moves away from the absorbing surface until some obstacle hinders its further movement.

B. *Attached cyprids*

As was described for planktonic barnacle cyprids, little increase was observed in either size or number of copper-containing vacuoles in parenchymatous connective tissue as the medium was enriched in copper ion. A trace of copper precipitate was found near the anterior pigment mass in some specimens where none was observed in animals of the same stage from normal sea water. The addition of copper ion to the medium was accompanied by a marked increase in the number and density of deposits in the hind-gut epithelium similar to that described for planktonic cyprids. Stomach secretion of copper, while increased over that

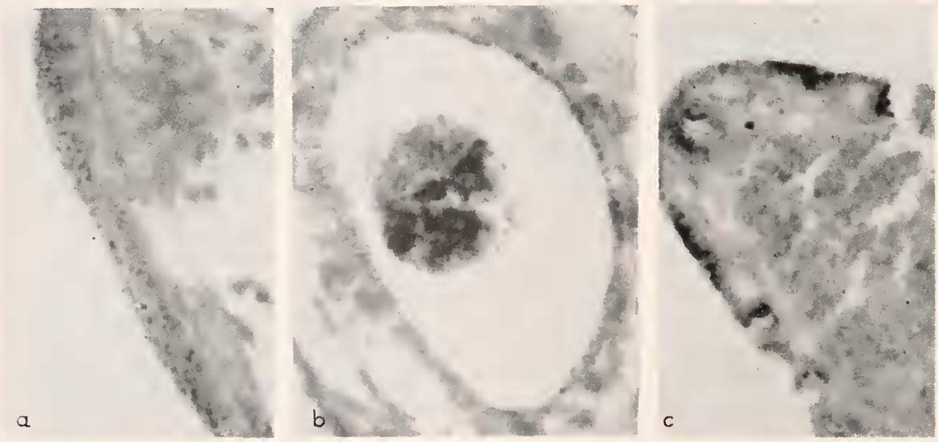

FIGURE 6. Loci of copper accumulation in attached cyprids exposed to excess ambient copper and stained with D.T.O. technique. (a) In the connective tissue underlying the cyprid carapace. (b) Within the food mass and in the secretory region of the stomach epithelium. (c) In the thoracic epidermal cells and in the secretory portion of the hind-gut.

shown by animals from this stage in normal sea water, was somewhat less than had been observed in the previous stage (Fig. 6).

If the rate of absorption of copper were proportional to its concentration within thoracic epidermal cells, then clearly less copper was taken up by cyprids after attachment than before. It is suggested that this reduced absorption results from decreased exposure of thoracic surfaces to sea water because the cirri are inactive during this stage and the mantle circulation is correspondingly reduced.

Sites of heavy copper concentration in the planktonic cyprid, such as the ventrolateral surfaces of the anterior part of the thorax, show somewhat less copper deposition in attached barnacles. The dorsal surface of the posterior half of the thorax, where the gut approaches the surface most closely, contained a fairly heavy concentration of copper (Fig. 6c). Some copper was present in the epithelium lining the fore-gut. The mouth parts and anal flaps contained considerably less copper than in the previous stage. Where precipitated copper was scant in the planktonic stage, *e.g.*, the mid-thorax and the limb folds, none was found in the attached animals.

The distribution of copper in cells underlying the carapace resembled that already described for planktonic cyprids exposed to a high ambient copper concentration, both in the early phases where epidermal and cuticular layers are in normal contact and later when they become separated (Fig. 6a).

C. *Decorticated settlers*

Isolated particles of precipitated copper were seen in some sections of normal animals in this stage. When the copper concentration of the medium was increased to 200 ppm, there was no increase in detectable copper in the parenchymatous sheath characterizing animals in this stage. In the enriched medium, however, the cylindrical cell mass, previously described (Bernard and Lane, 1961), contained deposits of copper (Fig. 7b). These precipitates were confined to

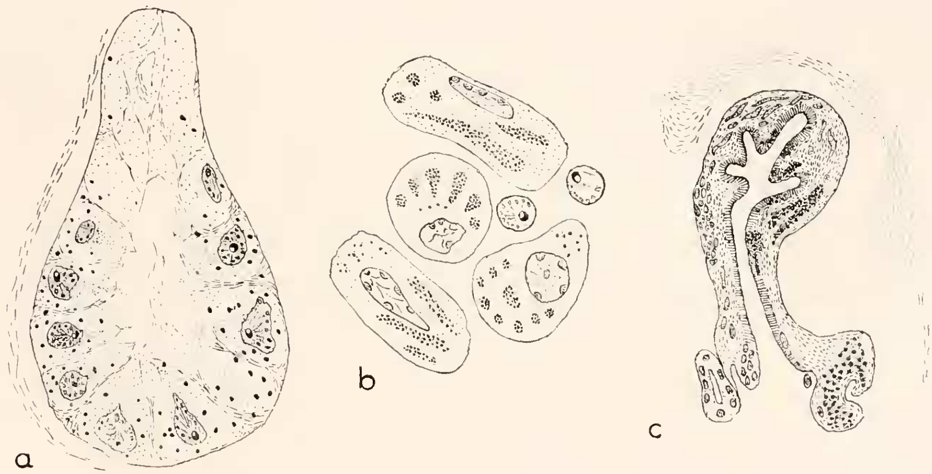

FIGURE 7. Regions of copper ion concentration in later phases of the decorticated stage by barnacles exposed to 200 p.p.m. Cu^{++} for one hour. (a) In the cytoplasm of peripheral organelle cells. (b) In fascicular vacuoles of the cylindrical cells. (c) Within one lobule of the glandular labyrinth.

fascicular inclusions within the cytoplasm of the cylindrical cells and were noted only in the latest phases of this stage when cell orientation is haphazard.

We suggested previously that inclusions within these cells are reservoirs for histolyzed material to be employed in subsequent histogenesis and organogenesis. The deposition of copper in these inclusions implies a dynamic exchange with the ambient sea water and directs attention to a possible role of these cells in concentration of other ions from sea water during morphogenesis. Vital staining experiments also indicated a rapid exchange between parenchyma vacuoles and the medium during this stage.

During the same phase at which these deposits were noted, but not in earlier specimens, some copper was detected in the cytoplasm of the cells in the peripheral organelles. This material was perinuclear in distribution, but revealed no consistent orientation with respect to the cell surface. (See Figure 7a above.) At the same time copper secretion was observed in a few cells of a tubule connected with the large mucin-secreting organ, believed to be the primorium of the gut.

In the glandular labyrinth, a very small compound tubular gland was found to be actively secreting copper. The D.T.O.-copper precipitate appeared in the gland epithelium and in irregular blotches in the subepithelial connective tissue, as well as in the cells of a finger-like projection which marked the juncture of the gland with the main organ (see Fig. 7c).

In view of the general characters of this stage, *viz.*, no ingestive apparatus and development within a closed gelatinous sheath, it appears that copper absorption occurs only by diffusion from the medium. The lack of localized deposits in specimens from normal sea water suggests that copper ion is not essential to physiological processes characteristic of this stage. The presence of particulate deposits of copper during only one phase of development strongly suggests that free exchange with the ambient medium takes place during a restricted period of development. This period may include concentration of other ions. Activity of certain cells or cell groups, in the absence of a clear general pattern, in either secreting or absorbing copper may be a vestigial functional character from a previous stage, or may presage future activity of these cells.

D. and E. *Young barnacles*

In the transition from the decorticated larva to the young barnacle the parenchymatous sheath becomes a stiff supportive casing for the organism. The homogeneous mass of connective tissue cells and fibers is clothed by epithelial layers on inner and outer surfaces. The inner epithelium consists of squamous cells and the outer covering epithelium is at first cuboidal. The epithelial surfaces

FIGURE 8. Presence of copper precipitate during the early period of shell formation in a specimen exposed to excess copper ion and treated with D.T.O. Note accumulation within the long cells penetrating the forming shell and in certain areas of the glandular region of the thorax.

are interconnected by protoplasmic extension of intermediate cells; the nuclei of these cells generally form a central layer. Within the cytoplasm of these long cells the copper precipitate was found in D.T.O.-treated specimens of the young stage before compartmental plates appeared (Fig. 8). Continued development results in a thickening of these radial cells and formation of lateral extensions from them, until a network of interlacing cytoplasmic strands unites the two epithelia. The copper deposit is no longer present after the strands have thickened. From these facts we assume that these cells are active in the absorption of solutes from sea water into the organism during this last phase of metamorphosis. It is possible that the appearance of copper in these areas at this stage is coincidental with active concentration of other ions; for example, calcium must be concentrated from sea water at this time to form the calcareous plates of the adult barnacle.

The cells in contact with the substrate form a stratified epithelium within whose lowest layer some copper is deposited.

Posterior to the stomach of the young barnacle a series of branching tubules develop which appear to be digestive caeca. In several specimens studied a copper deposit was discovered, but always in only one, or at most two, lobes of this organ. This phenomenon was noted in animals both with and without compartmental divisions.

Summary and Conclusions

1. Sites of copper ion concentration in barnacle cyprids from normal sea water, both planktonic and settled, suggest that the barnacle absorbs copper from the medium through permeable surfaces and eliminates excess copper by excretion through the epithelia of the hind-gut. Reservoirs within which copper storage may occur have been identified. A metabolic role is suggested for copper in normal physiology of barnacle settling stages.

2. Copper deposits are consistently absent from both decorticated settlers and from two stages of the young adults.

3. Exposure of planktonic cyprids and early settled forms to a high-copper medium resulted in increased rate of elimination from the gut epithelium, and identified various surface areas of the thorax as absorption sites. Transport of copper ions in solution from absorption surfaces to points of excretion is probably by way of circulating body fluids.

4. Copper deposits were observed in decorticated settlers only during later phases of this stage and in areas previously identified as active points of exchange with ambient sea water.

5. Absorption of copper from sea water containing excess copper during the stages of shell formation occurs via single-cell extensions through the thickness of the forming shell. Elimination of copper through a lobe of the digestive caecum was also noted during this period.

6. Copper localization studies on post-decortication stages suggest that copper absorption is coincident with absorption of other ions, particularly calcium, which is highly concentrated during these developmental periods.

7. Copper absorption during the cyprid-form developmental stages occurs through respiratory surfaces. It is suggested that the anti-fouling effectiveness of copper is due to interference with normal respiratory exchange.

8. The effect of the copper-ion concentration in the ambient medium on oxygen uptake of cyprids is currently being investigated and will form the substance of a subsequent communication.

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